Stereospecific synthesis of selected triglycerides: comments on acyl migration and analysis of configuration

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Received 15 April 1994; revision received 17 June 1994; accepted 17 June 1994

Abstract

Symmetrical and stereospecifically defined triglycerides have been prepared from racemic and (*R*)- and (*S*)-glycidol, respectively. The synthetic sequence allows the incorporation of sensitive fatty acids such as linolenic acid. Justification for retention of configuration and absence of acyl migrations during the synthesis is provided by the ^{1}H-NMR spectra of triglycerides that are replaced at the *sn*-1 and *sn*-3 positions with enantiomers of α-methoxytrifluoromethylphenylacetic acid (*bis*-MTPA esters). Partial hydrolysis of synthetic symmetrical triglycerides catalyzed by the lipase of *Rhizopus oryzae* was used to demonstrate the homogeneity of these preparations.

Keywords: Acyl migration; α-Methoxytrifluoromethylphenylacetic acid (bis-MTPA esters); *Rhizopus oryzae*; (*R*) and (*S*) glycidol

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Abbreviations: The following are for fatty acids: C, capric acid (decanoic acid, C10:0); L, linoleic acid (*cis,cis*-9,12-octadecadienoic acid, C18:2); La, lauric acid (dodecanoic acid, C12:0); Ln, linolenic acid (*cis,cis,cis*-9,12,15-octadecatrienoic acid, C18:3); M, myristic acid (tetradecanoic acid, C14:0), 0, oleic acid (*cis*-9-octadecenoic acid, C18:0); P, palmitic acid (hexadecanoic acid, C16:0); S, stearic acid (octadecanoic acid, C18:0). HPLC, high-performance liquid chromatography; MTPA, α-methoxytrifluoromethylphenylacetic acid; NMR, nuclear magnetic resonance.

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SSDI 0009-3084(94)02353-7

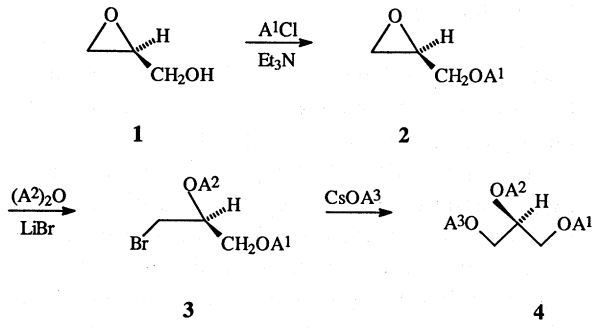
1. Introduction

Recently, a convenient synthesis of configurationally pure triglycerides was reported that was initiated with (*R*)- and (*S*)-glycidol [1]. The configurational purity of the products was, within experimental error, the same as that of the commercially available glycidol (92% ee). We wished to test this route further with air-sensitive fatty acids as well as triglycerides bearing medium-chain fatty acids at the *sn*-2 position. Such compounds are sought for research targeted to restructured tri-

glycerides and for studies related to human health. Evidence to support the retention of configuration for the chiral carbon of glycidol in the chiral triglycerides prepared is provided by an evaluation of the ^{1}H-NMR spectra of triglycerides that were synthesized from chiral glycidol and two equivalents of the chiral derivatizing agent, α-methoxytrifluoromethylphenylacetic acid (MTPA), also known as Mosher's acid [2]. Additional evidence for the constitution of the subject triglycerides was obtained by adapting recent work dealing with the characterization of diglyceride configuration. Seminal research using chiral isocyanates to convert diglycerides into diastereomeric carbamates that can be separated by high-performance liquid chromatography (HPLC) [3] has been employed to characterize the stereoselectivity in hydrolysis of triglycerides catalyzed by lipases (triacylglycerol hydrolases) [4]. Complementary research reported a critical evaluation of the utility of this analytical method for determining the configuration of specific triglycerides by using ethyl magnesium bromide to produce diglycerides that were then derivatized with an analogous chiral isocyanate [5]. A very 1,3-selective lipase from the fungus *Rhizopus oryzae* was used by us to generate diglycerides from the synthesized triglycerides. The partial glycerides were then converted to diastereomeric carbamates using (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate, and analysis used to verify structure.

2. Results and discussion

Both symmetrical and unsymmetrical triglycerides were prepared according to Scheme 1. The preparation of symmetrical triglycerides was initiated from racemic glycidyl oleate (±**2**) that was allowed to react with a fatty acid anhydride and lithium bromide to give a bromodiester (±**3**) [1]. Displacement of the bromide with cesium oleate yielded the 'OXO' type of triglyceride, where 'X' is a fatty acid other than oleic acid (Table 1) and for which the analytical discussion is deferred.



Scheme 1. Synthesis of triglycerides from glycidol, exemplified with (*S*)-glycidol. A^1, A^2 and A^3 are fatty acyl groups.

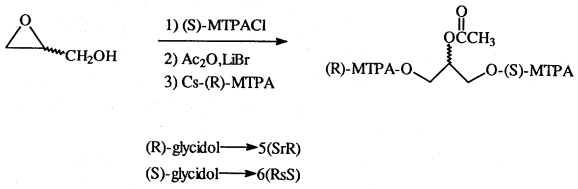
Table 1
Synthetic triglycerides[a]

4a OCO	**4e** (*R*)- and (*S*)-LaOO
4b OLO	**4f** (*R*)- and (*S*)-SOO
4c OMO	**4g** (*R*)- and (*S*)-SOL
4d OPO	**4h** (*R*)- and (*S*)-SOLn

[a]The abbreviations for the fatty acids are listed at the beginning of the paper.

(*R*)- and (*S*)-glycidol are available in 96% configurational purity (92%ee) and served as the starting material for the other triglycerides. The sequence of reactions was the same; the most sensitive fatty acid was used in the last step of the sequence. Thus, for example, (*S*)-(−)-glycidol, **1**, was converted to its stearic acid ester, **2** (A^1 = stearoyl), and the epoxide ring was opened with oleic anhydride/LiBr to give **3** (A^2 = oleoyl) and converted with cesium linolenate to (*S*)-**4h** (A^3 = linolenoyl), *sn*-LnOS. Each bromodiester and triglyceride was purified to homogeneity (TLC) by flash chromatography and characterized spectrally (Materials and methods).

Justification for the configurational purity of the compounds made by this route had been assessed originally [1] by treating the enantiomeric glycidols with a chiral carboxylic acid, namely α-methoxytrifluoromethylphenylacetic acid, MTPA. The ^{1}H-NMR spectra of diastereomeric glycidyl



Scheme 2. Preparation of *bis*-MTPA derivatives from glycidol.

esters were distinguishable at the CH_2 bearing the chiral acid residue. These spectral differences persisted in the bromodiesters and also in the final triglyceride that still bore the MTPA group on the original primary alcohol. In an additional exercise, the (*R*)-(+)-glycidol ester of (*S*)-(−)-MTPA was allowed to react with acetic anhydride/LiBr, and the resultant bromodiester was treated with the cesium salt of (*R*)-(+)-MTPA to yield **5** (Scheme 2; Fig. 1), which has the **SrR** configuration in which the letter designators for configuration have been ordered by *sn*-nomenclature (*sn*-1 carbon has the S-(−)-MTPA group attached). The central carbon of the *bis*-MTPA derivative becomes a pseudo-asymmetric center [6], and the compound is diastereomeric with **6** that was obtained by initiating the sequence with (*S*)-glycidol. Alternatively, the reversal of the order of attachment of the two enantiomeric MTPA residues to (*R*)-(+)-glycidol would have accomplished the same task. The ^{1}H-NMR spectra are clearly differentiated, and each of the two diastereotopic protons on the primary carbons can be distinguished (Fig. 1). The H_A's of each diastereomer are equivalent, as are the H_B's, but the sets of one diastereomer can be discerned from those of the other. Each diastereomer indicates the presence of a few per cent of the other diastereomer, and this is ascribed to the presence in each enantiomer of the original glycidol itself of ~4% of the opposite stereoisomer. By contrast, the (*R,R*)- and (*S,S*)-stereoisomers of the *bis*-MTPA esters (not shown)

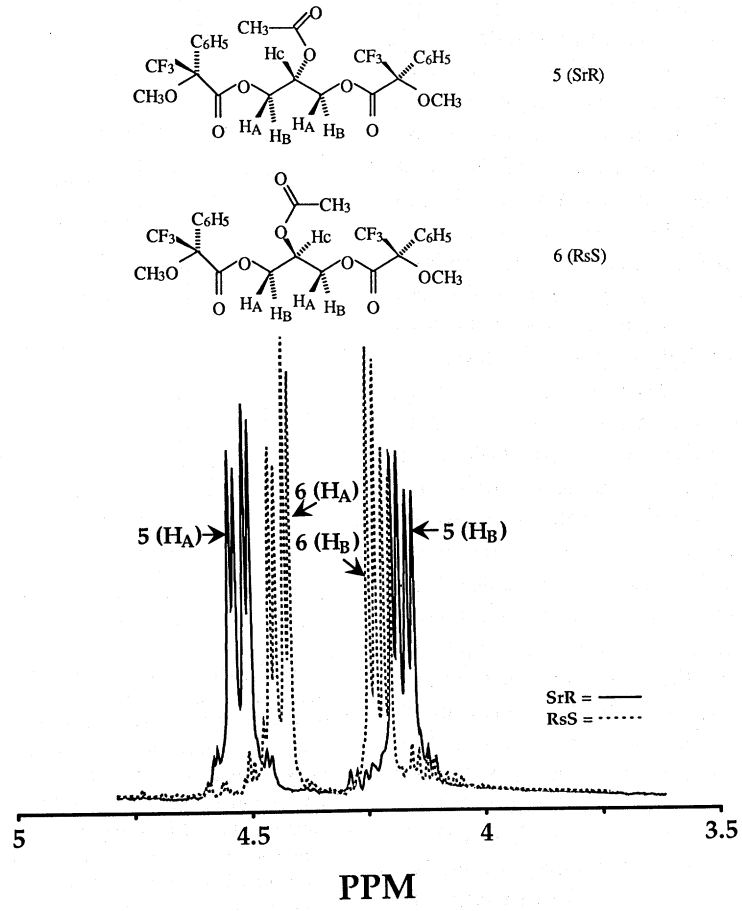


Fig. 1. Portion of the ^{1}H-NMR spectrum indicating diastereomeric purity of the *bis*-MTPA derivatives **5** and **6**.

were prepared using the (*R*)- or (*S*)-MTPA units twice, respectively. The central carbon in these compounds is not chiral, and the methylene proton signals are more complex, since all four methylene protons of each enantiomer are non-equivalent. There is, therefore, no indication of loss of stereochemical integrity during the course of these transformations.

Recently, an extremely useful procedure for determining lipase stereoselectivity in reactions with homogeneous triglycerides was reported [3] that also has utility in determining triglyceride structure. The method involves partial hydrolysis to diglycerides that are then converted to diasteromeric carbamates with (*R*)- or (*S*)-1-phenylethyl isocyanate for HPLC analysis. This idea was adapted for evaluating triglyceride structure using ethyl magnesium bromide as a non-stereoselective reagent to generate diglycerides [5]. Because acyl migration is quite facile during cleavage caused by the organometallic, the conditions for this procedure are critical. In addition, reaction occurs readily at the *sn*-2 position, further complicating product mixtures. The *Rhizopus* genus of fungus produces lipases that are quite 1,3-positionally selective and can be employed in partial hydrolyses at pH 7.0 and 25°C for reaction times up to 4 h without any noticeable acyl migration [7]. Reaction with a triglyceride of the form OXO would produce diglycerides *sn*-OX(OH) and *sn*-(OH)XO. Any O(OH)O would be caused presumably by direct removal of X from the 2 position, and any *sn*-O(OH)X and *sn*-X(OH)O would derive from acyl migration. Stereoselectivity may be observed in this analytical protocol but would not interfere with structure determination of this type of triglyceride. A commercial sample of 1,3-diolein that contained the 1,2-isomer was reacted with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate; the chromatogram shows the 1,3- and diastereomeric 1,2-adducts (Fig. 2A). In order to avoid possible fractionation of derivatives, no intermediate chromatographic purification of the derivatization mixture was employed. Hence, the unreacted isocyanate is present but does not interfere with the interpretation of the chromatograms. Triolein was employed then to develop a useful procedure for the projected enzymatic hydrolysis. At 62% conversion (based upon only two equivalents of fatty acid available) the major diglycerides were the enantiomeric 1,2 (2,3)-dioleins. Only 4% of the 1,3-diolein was formed (data not shown). Since commercial lipases are not pure proteins, the

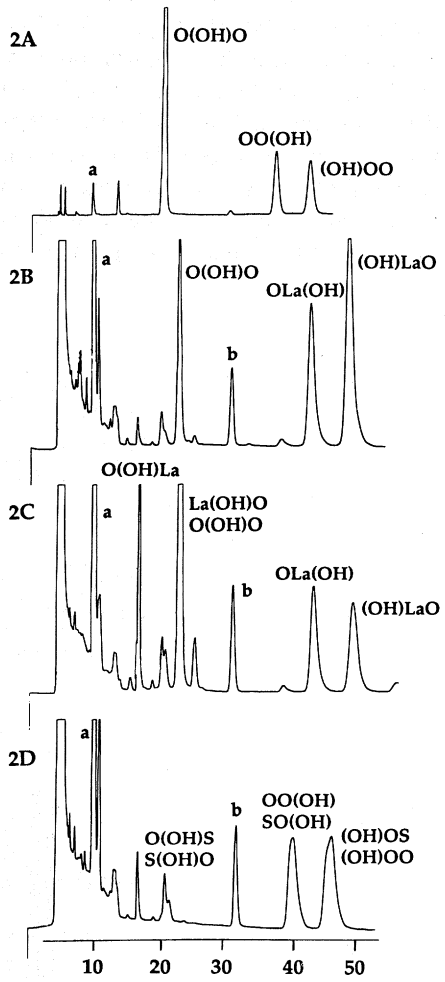


Fig. 2. HPLC separations of carbamate derivatives of diglyceride on silica gel with 0.5% 2-propanol/hexane at 1.0 ml/min and UV = 280 nm. Peak "a" = unreacted isocyanate. The urea formed from the isocyanate as a byproduct eluted at about 70 min. A. Derivatives formed from a commercial sample of 1,3-diolein containing the 1,2-isomer. B. Partial lipolysis of synthetic OLaO followed by derivatization. C. Diglycerides of sample B treated with acid to cause acyl migration and then derivatized. D. Superimposed chromatograms of the derivatives formed with the partial lipolyses products of *sn*-SOO and *sn*-OOS.

hydrolysis at the 2 position could be explained by the adventitious presence of a less selective hydrolase. Whatever its basis, the loss of fatty acid at the 2 position is most easily justified as direct hydrolysis. The lipolysis of triolein was somewhat biased, producing more of the 2,3-diolein; i.e. the *sn*-1 position had reacted faster, as was shown previously for *Rhizopus arrhizus* lipase [4].

Partial hydrolysis of OLaO (La = lauric) by *Rhizopus oryzea* lipase followed by derivatization produced *sn*-OLa(OH) and *sn*-(OH)LaO, with the latter predominant, as well as 19% O(OH)O for a sample that had been hydrolyzed to 48% conversion (Fig. 2B). Although this point requires more methodical investigation and is beyond the scope of this report, it appears that 1,3-positional selevtivity may hinge upon the identity of the acid residue in the 2 position. Brief treatment of the crude diglycerides in ether containing a trace of *p*-toluenesulfonic acid produced a desired 1,2-(2,3-)acyl migration. The carbamate derivatives that were subsequently obtained allowed identification of *sn*-O(OH)La and *sn*-La(OH)O (Fig. 2C). These were absent in the original hydrolysate of OLaO, ensuring the absence of acyl migration during the synthesis of the triglyceride, during exposure to hydrolytic conditions, and during the product work-up and derivatization!

The procedure of Rogalska et al. [3] unfortunately cannot be used for triglycerides of the type YXY′ where Y and Y′ have the same chain length. The derivatives of *sn*-YX(OH) and *sn*-Y′X(OH), for example, are not separated by silica gel HPLC. Thus partial lipolysis of *sn*-OOS and *sn*-SOO, (*R*)- and (*S*)-**4f**, respectively, produced diglycerides the carbamates of which could not be distinguished by HPLC (Fig. 2D). Thus the configurational purity of **4e–h** rests on analogy provided by the analyses of MTPA derivatives and **4a–d**. It should be noted, however, that racemization during the synthetic sequence and during hydrolysis could only occur through 1,3-acyl migration. This phenomenon has been evaluated and is much less probable than 1,2-acyl migration [8]. Parenthetically, since the constitution of *sn*-SOO and *sn*-OOS are assured, their reactions catalyzed by a lipase allow an assessment of the stereoselectivity of the catalyst according to the fatty acids present on the attacked position. The hydrolyses again favored the *sn*-1 position, though only slightly, and did not indicate any preference for one fatty acid over another for these two acids.

In conclusion, the synthetic method can be employed to incorporate polyunsaturated fatty acids in specifically constructed triglycerides. The methodology for characterizing the stereochemistry of a triglyceride [5], or stereoselectivity of a lipase [4], has been improved (Materials and methods) through the observation that 1,2-diglycerides do not undergo 1,2-acyl migration even at 85–90°C in toluene. This allows a much shorter derivatization time. Additionally, a base catalyst is not required for the condensation reaction. Also, the use of 2-propanol and hexane as an HPLC solvent seems more convenient and less expensive than solvent mixtures used to date.

3. Materials and methods

3.1. General procedures and reagents

Melting points are uncorrected and were performed on a Fisher-Johns Hot Stage apparatus. Silica gel TLC plates of 0.25 mm thickness from Merck & Co. were used to monitor reactions and chromatographic separations with iodine vapor for visualization. Aldrich Chemical Co. silica gel (230–400 mesh, 60 Å) was employed for flash chromatography [9]. Solvents were reagent grade or better and were obtained from Aldrich, Burdick and Jackson, and Mallinckrodt Companies. All reagents were purchased from Aldrich, including the two chiral derivatizing agents discussed. Tetrahydrofuran (THF) was distilled from lithium aluminum hydride and stored over molecular sieves. Other reagents and solvents were employed directly. (*R*)-(+)-glycidol and its enantiomer were purchased from Aldrich as well and are 96% optically pure [10]. The lipase used was Amano Co. Lipase FAP (*Rizopus oryzae*), which has a label activity on olive oil of 150 μmol FFA/min/mg of powder.

Gas-liquid chromatography (GLC) was performed with a Chrompack-Packard model 438A instrument fitted with a Supelco SP2330 capillary column (0.25 mm ID × 30 m) using helium carrier

and a 50:1 split ratio and flame ionization detector.

Infrared data were recorded with a Perkin Elmer 1310 spectrophotometer using 1% solutions in CCl_4. NMR spectra (^{13}C and ^{1}H) were obtained with a JEOL JNM-GX400 FT-NMR spectrometer with $CDCl_3$ solvent and tetramethylsilane as reference. Mass spectra were obtained with a Hewlett-Packard HP-5995 GC/MS system employing an OV-1 column (0.25 mm ID × 30 m) and NH_3.

3.2. Synthesis of triglycerides

The preparation of triglycerides from glycidol has been described [1]. Purifications of intermediate bromodiesters, **3**, and the triglycerides, **4**, were accomplished using flash chromatography [9] with 2% ethyl acetate-hexane for **3** and 3% for **4** (Scheme 1). The following procedure for converting **3** (A^1 = stearoyl, A^2 = oleoyl) to *sn*-LnOS ([S]-**4h**) is convenient for handling fatty acids that are air-sensitive. Linolenic acid (1.20 g, 4.20 mmol) and Cs_2CO_3 (0.70 g, 2.10 mmol) were swirled in methanol (25 ml) until the carbonate had dissolved. The mixture was concentrated on the flash evaporator (35–40°C) and then placed in a vacuum desiccator at 0.1 Torr over KOH for 1 h. The bromodiester, (*S*)-3-bromo-1,2-propanediol, 1-stearate, 2-oleate (*m/z* = 703, 705 $[M + 17]^+$); (1.41 g, 2.10 mmol) was added to the dried cesium linolenate with 7 ml each of dried THF and hexamethylphosphoric triamide. The mixture was immediately placed under nitrogen and warmed at 55°C for 4–5 h. The mixture was acidified with cold 2N HCl and extracted with ether. Flash chromatography yielded *sn*-LnOS (1.20 g, 64.5%) as a colorless oil that was homogeneous by TLC (5% ethyl acetate-hexane) R_f = 0.17 (the bromodiester R_f = 0.32); IR 3020, 1740 cm^{-1}; ^{1}H-NMR δ 5.32 (m, 9H, HC=CH and $-OCH_2\underline{C}H\underline{C}H_2O-$), 4.27 and 4.21 (2dd, 4H, $J[H_A-H_B]$ = 11.8 Hz, $J[H_A-H_C]$ = 4.1 Hz, $J[H_B-H_C]$ = 6.1 Hz), 2.81 (bt, 4H, $=CCH_2C=$), 2.31 (t, 8H, J = 7.5 Hz), 2.03 (bm, 6H, CH_2CO_2-), 1.61 (bm, 6H, $CH_2CH_2CO_2-$), 1.26 (CH_2 env.), 0.98, 0.88 (overlapped t's, ca. 9 H, CH_3's) ppm; ^{13}C-NMR (diagnostic signals) δ 173.63, 173.24 (C = 0), 132.40, 130.66, 130.47, 130.12, 128.69, 128.22, 127.58 (C=), 69.38, 62.56 (C—O) ppm; *m/z* = 901 $[M + 18]^+$.

3.3. Mosher diesters (bis-MTPA esters) 5 (RrS) and 6 (RsS) (Scheme 2)

The cesium salt of (*S*)-(−)-MTPA was prepared with Cs_2CO_3 in methanol as described above, and then allowed to react with the appropriate bromodiester. For example, the (*R*)-(+)-MTPA ester of (*S*)-(−)-glycidol was treated with one equivalent of acetic anhydride and three equivalents of LiBr in benzene at ambient temperature overnight to form (*S*)-3-bromo-1,2-propanediol, 1-(*S*)-1′-methoxy-1′-trifluoro-methylphenylacetate, 2-acetate. This compound was isolated by diluting the benzene with ether and washing the organic phase with water. The organic phase was dried ($MgSO_4$), concentrated and then employed directly in the displacement step performed as described above. The resulting triglyceride, **5** (RrS), was purified by flash chromatography eluting with 10% ethyl acetate-hexane. The ^{1}H-NMR spectra of **5** and **6** were most clearly distinguished by the methylene protons (Fig. 1). For **5**: δ 4.49 and 4.15 (2dd, $J[H_A-H_B]$ = 11.8 Hz, $J[H_A-H_C]$ = 4.4 Hz, $J[H_B-H_C]$ = 5.6 Hz) ppm; for **6**: 4.44 and 4.24 (2dd, $J[H_A-H_B]$ = 11.9 Hz, $J[H_A-H_C]$ = 4.4 Hz, $J[H_B-H_C]$ = 5.4 Hz) ppm. Signals for OCH_3 (s,3.43 ppm) and acetate methyl (s, 1.89 ppm) were not distinguishable.

3.4. Characterization of triglycerides by enzyme-catalyzed hydrolysis

The following procedure is typical: triglyceride **4b**, OLaO (about 0.5 mmol) was carefully weighed into a 2 ml volumetric flask and diluted to 2.0 ml with isooctane. Aliquots (500 μl) were placed into two reaction vessels, and 5.0 ml of a solution of 0.30 g of *R. oryzae* lipase powder dissolved in 15 ml of 0.05M phosphate buffer (pH = 7.0) was added to each mixture. The samples were swirled (30°C, 170 rev/min) for 1 h. One sample was titrated to pH = 11.0 with standard NaOH and corrected with a blank to obtain % conversion [11]. The other sample was shaken with 3 × 15 ml of hexane. The extract was washed with water,

dried (Na_2SO_4) and concentrated at $<40°C$. A 50 μl aliquot of crude material was dissolved in a vial containing 0.5 ml of toluene, and 60–70 μl of (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate was added. The vial was capped and heated at 90°C for 1 h. The toluene was stripped using heptane as a chaser, since toluene could interfere with UV detection of the derivatives. The warm residue was triturated with 2 × 1 ml of hexane to remove much of the solid urea that forms by reaction of excess isocyanate. The hexane solution was filtered through a plug of Na_2SO_4 and cotton, and analyzed directly (Fig. 2).

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